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Activation of Bruton's Tyrosine Kinase (BTK) by a Point Mutation in Its Pleckstrin Homology (PH) Domain

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Summary

Bruton's tyrosine kinase (BTK) is a nonreceptor tyrosine kinase critical for B cell development and function. Mutations in *BTK* result in X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice. Using a random mutagenesis scheme, we isolated a gain-of-function mutant called *BTK whose expression drives growth of NIH 3T3 cells in soft agar. *BTK** results from a single point mutation in the pleckstrin homology (PH) domain, where a Glu is replaced by Lys at residue 41. BTK* shows an increase in phosphorylation on tyrosine residues and an increase in membrane targeting. Transforming activity requires kinase activity, a putative autophosphorylation site, and a functional PH domain. Mutation of the SH2 or SH3 domains did not affect the activity of BTK*. Expression of BTK* could also relieve IL-5 dependence of a B lineage cell line. These results show that transformation activation and regulation of BTK are critically dependent on the PH domain.**

Introduction

Nonreceptor protein tyrosine kinases (reviewed by Bolen, 1993) are key regulators of the development and function of lymphocytes. The structure of these kinases is characterized by the Src homology (SH) tyrosine kinase domain and additional SH domains, including SH2 and SH3, which serve as protein–protein interaction sites (reviewed by Pawson and Gish, 1992).

BTK belongs to a new subfamily of nonreceptor tyrosine kinases, which includes Tecl (Mano et al., 1990), TecII (Mano et al., 1993), Itk (Siliciano et al., 1992), and DSrc28C (Gregory et al., 1987). *BTK* was recently identified as the defective gene in human X-linked agammaglobulinemia (XLA) (Tsukada et al., 1993; Vetrie et al., 1993) and murine X-linked immunodeficiency (xid) (Rawlings et al., 19 Thomas, et al., 1993). In XLA patients, there is less th 1% of normal levels of mature B cells in the periphe blood and immunoglobulin levels are drastically reduc In xid mice, B cell numbers are reduced to around 5(of normal and certain immunoglobulin subclasses, su as immunoglobulin M (IgM) and IgG3, are greatly creased. B cells from xid mice are unresponsive to terleukin-5 (IL-5; Koike et al., 1995; Hitoshi et al., 19 IL-10 (Go et al., 1990), CD38 (Yamashita et al., 1995; H ard et al., 1993), and CD40 (Hasbold and Klaus, 19 Faris et al., 1994) stimulation. This suggests that BTK n be critical for multiple pathways important in B cell fu tion. Recent work has demonstrated that IL-5 (Sato et 1994) or IL-6 (Matsuda et al., 1994) stimulation, and I receptor (Saouaf et al., 1994) or FcεRI (Kawakami et 1994) cross-linking all lead to BTK activation.

BTK contains SH2 and SH3 domains, which have b shown to be important for signal transduction in many r receptor tyrosine kinases. Binding to tyrosine-phospl ylated or nontyrosine-phosphorylated proteins can be diated through SH2 units (Pawson and Gish, 19 Pendergast et al., 1991). SH3 domains interact with line-rich motifs (Koyama et al., 1993). Proline-rich regi of BTK were found to interact with the SH3 domain the Src family kinases in vitro, but not in vivo (Chen al., 1994). The importance of these interactions aw further functional studies.

The most distinctive feature of the BTK family of tyro kinases is the presence of a pleckstrin homology (PH) main in its amino-terminal region. PH domains are fo in over 70 proteins involved in signal transduction cytoskeletal structures (Haslam et al., 1993; Mayer et 1993; reviewed by Musacchio et al., 1993; Gibson et 1994). The PH domain is approximately 120 aa long can be divided into six subdomains. The structure of domains determined by nuclear magnetic resonance pleckstrin (Yoon et al., 1994) and β-spectrin (Macia al., 1994) as well as the crystal structure of the PH dor of dynamin (Ferguson et al., 1994) have been solved. spite the low amino acid identity among PH domains different molecules, the three dimensional structure PH domains are highly conserved. The core of the domain consists of seven anti-parallel β sheets and a boxy-terminal region folded into an α helix.

Although the precise function of the PH domain is known, several lines of evidence suggest a critical rol it in BTK function. The xid mutation, R28C, and se XLA mutations (Bradley et al., 1994; de Weers et al., 1 are located within the PH domain. The carboxy-tern region has been shown to interact with the β/γ sub of trimeric G proteins (Gβγ) (Tsukada et al., 1994; Tou et al., 1994). Trp124 of BTK, the only conserved res among all PH domains, is required for this Gβ/γ interac Also, the amino-terminal region of the PH domain of p strin has been shown to bind to phosphatidylinositol

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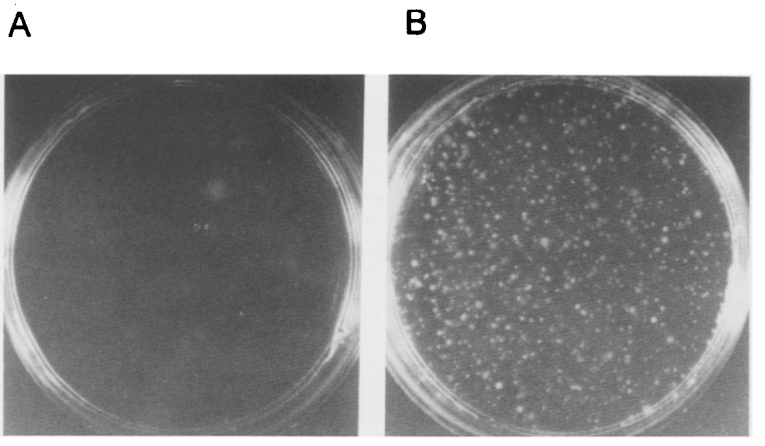


Figure 1. Transformation of NIH 3T3 Cells by BTK*

NIH 3T3 cells were infected with the following MuLV rescued genes: A, wild-type *BTK* gene (left); B, *BTK** gene (right). After G418 selection for 2 weeks, 10^4 cells were plated in soft agar per 6 cm plate and colonies were photographed 15 days after plating.

bisphosphate (PIP_2) (Harian et al., 1994), suggesting that lipid modification may regulate PH domain function. Finally, the PH domain of BTK can associate with isoforms of protein kinase C (PKC) in mast cells and B cells (Yao et al., 1994) implying a role for the PH domain in additional interactions.

Study of gain-of-function mutants has been a useful approach to investigate signal transduction pathways. An activated mutant of mitogen-activated protein kinase (MAPK) in Drosophila allowed placement of MAPK downstream of *torso* and *sevenless* (Brunner et al., 1994). Similar approaches have been used to dissect signal transduction pathways in yeast and Caenorhabditis elegans (Brill et al., 1994; Ruvkun et al., 1991). Nature has provided us with many loss-of-function mutants of *BTK*, but no gain-of-function mutants. We sought mutations that would result in BTK activation using a retroviral passage mutagenesis and cellular selection scheme scoring for a transformed phenotype. Analysis of the BTK* mutation shows the critical role of the PH domain in the regulation of the BTK family of tyrosine kinases and provides an important genetic tool for the study of B lymphoid development.

Results

Isolation of an Activated Mutant of BTK

Many activated tyrosine kinases are capable of transforming NIH 3T3 cells as measured by growth in soft agar. Wild-type BTK was not oncogenic in this assay (data not shown). We exploited the high mutational rate of the retroviral life cycle to screen for transforming mutants of *BTK* (Goga et al., 1993). NIH 3T3 cells were infected with a helper-free retrovirus stock expressing a wild-type *BTK* clone and plated in soft agar. A few rare colonies appeared and were picked and expanded in liquid culture. To confirm that the transformed phenotype of these colonies was due to mutations in *BTK* rather than secondary cellular events, we used a replication-competent helper virus, Moloney murine leukemia virus (MuLV), to rescue the integrated retroviral *BTK* gene from the transformed cells. One of the rescued *BTK* constructs demonstrated transforming activity upon infection of new NIH 3T3 cells (Figure 1), demonstrating that the transformed phenotype was associated with a mutation in the retroviral genome.

A Point Mutation in the PH Domain of BTK Leads to the Transformed Phenotype

Immunoblotting analysis did not show an obvious change in the size of the BTK protein. To determine the precise mutation, a phage genomic DNA library was constructed from the transformed cells and screened for the integrated retroviral construct whose *BTK* insert was sequenced. A single point mutation of G to A at nucleotide position 257 resulted in a change of glutamic acid at position 41 to lysine (Figures 2A and 2B). This residue is located in the third subdomain of the PH domain and is identical in all members of the BTK family of tyrosine kinases. We named this transforming allele *BTK**.

To demonstrate unequivocally that the transforming phenotype was due to this single point mutation in BTK, a chimeric construct was made by fusing the first 173 aa from BTK*, including the E41K mutation, with the rest of the wild-type BTK sequence. This construct transformed NIH 3T3 cells, indicating that the point mutation E41K in the PH domain of BTK is sufficient to activate the transforming potential of BTK.

BTK* Shows Increased Tyrosine Phosphorylation

Activation of a tyrosine kinase is often associated with increased kinase activity, increased tyrosine phosphorylation, or both. Wild-type BTK and BTK* have comparable in vitro autophosphorylation kinase activities (Figure 3A). Anti-phosphotyrosine immunoblotting of wild-type BTK and BTK* showed that BTK* was highly phosphorylated on tyrosine in vivo (Figure 3A). Equal amounts of BTK protein were analyzed in these studies as monitored by anti-BTK immunoblotting. Following in vivo labeling with ^{32}P-orthophosphate and recovery by immunoprecipitation, phosphoamino acid analysis demonstrated that BTK* has increased phosphorylation on Tyr and Ser residues (data not shown).

Increased Membrane Targeting of BTK*

Membrane association is often correlated to nonreceptor

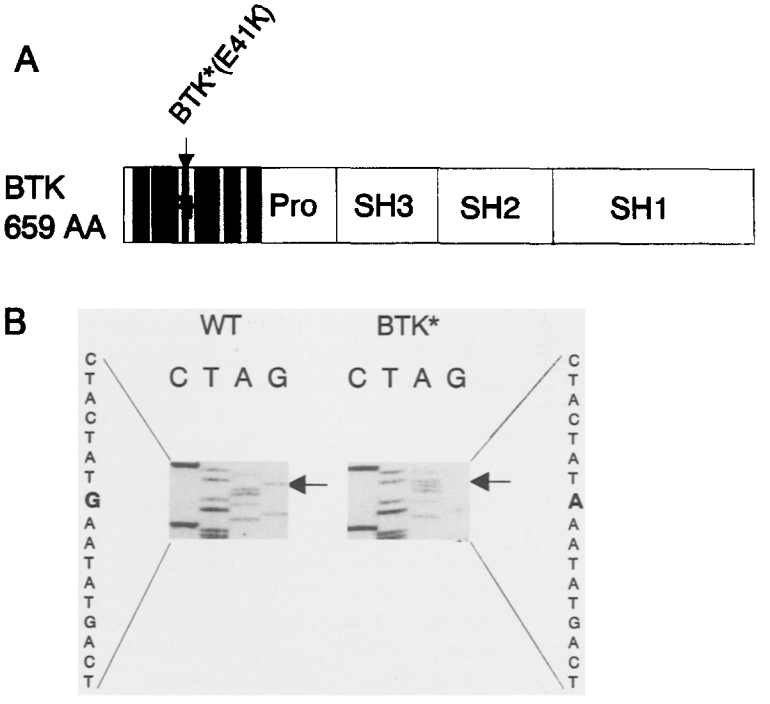


Figure 2. A Point Mutation Found in the Domain of BTK*

(A) A linear representation of the BTK pro sequence. Domains of BTK are represente follows: SH1, the kinase domain; SH2, the homology 2 domain; SH3, the Src homolo domain; Pro, the proline rich stretch; PH, pleckstrin homology domain. The sha boxes represent six subdomains of the PH main. The position of BTK* mutation is i cated by an arrow.

(B) Sequencing ladder showing the *BTK** m tion. Double-stranded DNA sequencing ar sis (Sanger et al., 1977) of the reconstru transforming allele of *BTK* (*BTK**) (right) wild-type *BTK* (left) showed a single nucleo substitution at position 257 as indicated by arrows.

tyrosine kinase activation. More than 95% of wild-type BTK was found in the cytosol in resting cells (Kawakami et al, 1994; Tsukada et al., 1993). NIH 3T3 cells stably expressing wild-type BTK or BTK* were fractionated by hypotonic lysis and Dounce homogenization. The amount of BTK protein in each fraction was measured by immunoblotting. There was a 3- to 5-fold increase of BTK* protein in the membrane fraction compared with wild type (Figure 3B). Samples were probed for p120GAP with an anti-GAP antibody to ensure that there was no cytosolic contamination of the membrane fraction. These results, together with the previous finding that BTK activation correlated with increased membrane association (Kawakami et al., 1994), suggest that BTK may function through reversible membrane association.

BTK* Oncogenic Activity Requires its Kinase Activity and a Putative Autophosphorylation Site

Independent pedigrees show that mutations in alternative domains of BTK can be associated with XLA (Bradley et al., 1994; Saffran et al., 1994; de Weers et al., 1994; Zhu et al., 1994; Vetrie et al., 1993). To determine the structural requirements for BTK* activation, we combined this BTK* mutation with secondary mutations in domains implicated in regulating BTK function. Inhibition of BTK* transformation by a secondary mutation would indicate that a specific domain is required to send a downstream signal from BTK*.

We first looked at mutations outside the PH domain. Retroviral constructs of wild-type *BTK* and *BTK** containing secondary mutations were made in parallel. The transformation activity of these constructs was scored by growth in soft agar (Figure 4A). The R307K mutation in the SH2 domain of BTK eliminates its ability to bind to tyrosine-phosphorylated proteins (D. Saffran, unpublished data). The K430R mutation abolishes kinase activity (c not shown). The Y551F mutation removes a putative a phosphorylation site of BTK. A deletion between resid 204 and 263 removes the SH3 domain. These mutant the context of either BTK or BTK* were introduced NIH 3T3 cells and protein expression was measurec anti-BTK immunoblotting (Figure 4B). The BTK(R3C and BTK*(R307K) mutants showed a lower level of exp sion compared with the other mutants. This may re from reduced protein stability, since another mutatio the SH2 domain of BTK has been shown to reduc stability (Saffran et al., 1994).

None of the single mutations except BTK* activate transformation in two independent experiments. Am the BTK* chimeras, BTK*(R307K) and BTK*(Δ204–: retained transformation activity, while BTK*(K430R) BTK*(Y551F) did not transform NIH 3T3 cells. Thes sults imply that the kinase activity and the putative a phosphorylation site, but not the SH2 and the SH3 mains, are required for efficient BTK* downstr signaling.

The PH Domain Is Essential for BTK* Signaling

To assess the functional role of the PH domain in B signaling, we examined the effect of secondary PH dor mutations on BTK* transformation. The R28C mut represents the mutation found in xid mice. The W mutation changes the uniformly conserved trypto found in the carboxy-terminal α-helical portion of PH mains. The W124G mutation was shown to abrogat association of the BTK PH domain with Gβ/γ (Tsuka al., 1994). These mutations were made in the conte wild-type BTK and BTK* (Figure 5A) and introduced

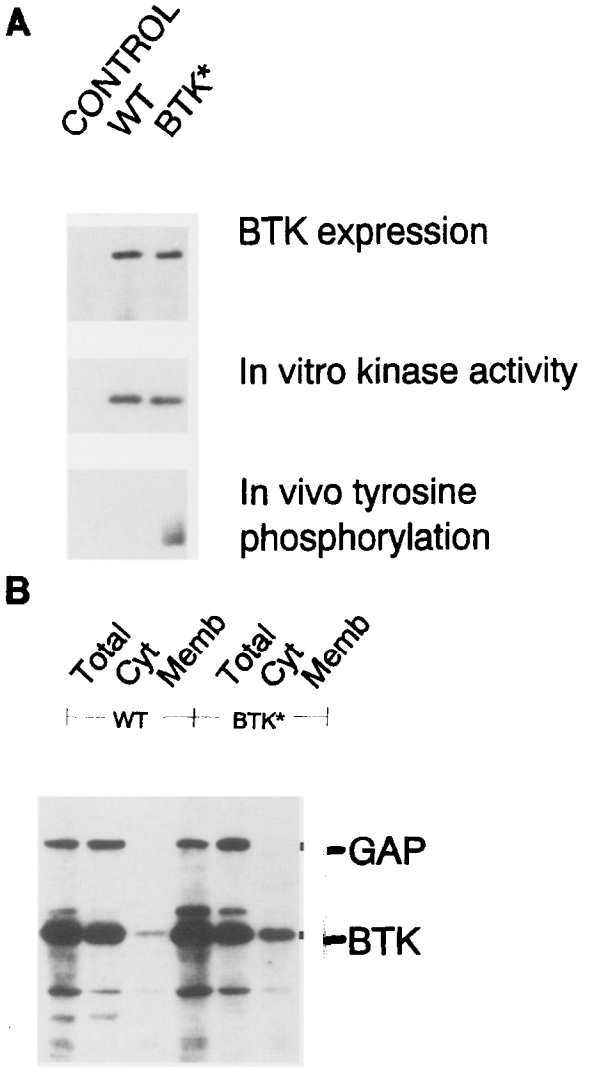


Figure 3. Cellular Tyrosine Phosphorylation and Localization of BTK*

(A) Increased tyrosine phosphorylation of BTK*. Cells expressing the TK–*neo* (control), wild-type BTK, or BTK* were lysed and BTK proteins were immunoprecipitated with anti-BTK antiserum. Half of the immunoprecipitates were washed and subjected to in vitro autokinase assay. The immunoprecipitates and the kinased samples were subjected to 10% polyacrylamide gel electrophoresis (SDS–PAGE). BTK proteins were immunoblotted with anti-BTK antiserum (1:500), followed by HRP-conjugated goat anti-rabbit secondary antibody (1:5,000), and visualized by ECL technique (Amersham) (top). The in vitro autokinase activity of BTK proteins were autoradiographed (middle). The in vivo tyrosine phosphorylation of BTK was measured by immunoblotting with anti-phosphotyrosine antibody 4G10 (1:1,000), followed by HRP-conjugated goat anti-mouse secondary antibody (1:5,000) and visualized by ECL technique (bottom).

(B) Increased membrane targeting of BTK*. NIH 3T3 cells expressing wild-type BTK and BTK* were fractionated by hypotonic lysis and Dounce homogenization as described (Kawakami et al., 1994). Total cell lysates, the cytosolic fractions, and the membrane fractions were analyzed by immunoblotting with anti-BTK antiserum (1:500) or anti-GAP antibody (1:250), followed by HRP-conjugated goat anti-rabbit secondary antibody (1:5,000), and visualized by ECL technique.

NIH 3T3 cells. BTK expression was measured by immunoblotting (Figure 5B).

Two independent experiments showed that these PH domain mutations did not transform NIH 3T3 cells by themselves and greatly reduced transformation activity in chimeras with BTK* (Figure 5A). These data suggest that other regions and functions of the PH domain are needed for BTK* downstream signaling.

Tyrosine Phosphorylation of BTK* Chimeras

The tyrosine phosphorylation state of BTK* chimeras was examined to determine whether it correlates with transformation activity. The in vivo tyrosine phosphorylation state of the BTK* chimeras was examined by anti-BTK immunoprecipitation followed by anti-phosphotyrosine immunoblotting. Equal amounts of BTK protein were analyzed as monitored by anti-BTK immunoblotting (Figure 6, top). The SH2 and SH3 domain mutations (BTK*[R307K] and BTK*[Δ204–263]) did not alter the hyperphosphorylation state of BTK* on tyrosine residue(s) (Figure 6, bottom). This correlated with their transforming activities. The kinase mutant chimera (BTK*[K430R]) was not hyperphosphorylated on tyrosine residue(s), suggesting that BTK kinase activity is responsible for the increased tyrosine phosphorylation. Mutation at the putative autophosphorylation site (BTK*[Y551F]) did not affect the hyperphosphorylation of BTK on tyrosine residue(s). This demonstrates that BTK* must be phosphorylated on tyrosine residue(s) other than the putative autophosphorylation site. Neither of the PH domain mutation BTK* chimeras were hypertyrosine phosphorylated. These combined results suggest that transformation by BTK* chimeras is associated with their increased tyrosine phosphorylation, but hyperphosphorylation per se is not sufficient for the transforming phenotype.

Role of BTK* in B Lymphocytes

Based on the genetic and biochemical findings that BTK is involved in the IL-5 signaling pathway (Sato et al., 1994; Hitoshi et al., 1993), we investigated the effect of BTK* on this B cell growth control pathway. The Y16 cell line (Takaki et al, 1990) was derived from an IL-5-dependent pro-B cell. Y16 cells were infected with equivalent titer ($\sim 10^6$ cfu/ml) retroviruses expressing wild-type *BTK*, *BTK**, or the v-*abl* oncogene, all containing a cis-linked neomycin-resistance gene (TK–*neo*). An additional control of a TK–*neo*-only vector was used. v-*abl* was chosen as a positive control because it is a strong tyrosine kinase oncogene that can produce factor independence for many hematopoietic cell types. Infected Y16 cells were selected with G418 for 10 days and then assayed for IL-5 independence by plating 2×10^4 cells per well on 96-well plates. Y16 cells expressing wild-type retroviral *BTK* could not grow in the absence of IL-5 (Figure 7A). Two separate preparations of BTK* virus gave rise to multiple IL-5-independent clones. V-Abl virus uniformly gave factor independent clones within 5 days. The lower frequency of growth factor–independent clones associated with BTK* suggests that *BTK** has a much weaker effect on Y16 cells than v-*abl* and may require a complementary cellular event to establish growth factor independence. IL-5-independent clones derived from BTK* infection were expanded and the expression of *BTK* from the retroviral genome in these clones was confirmed by S1 nuclease

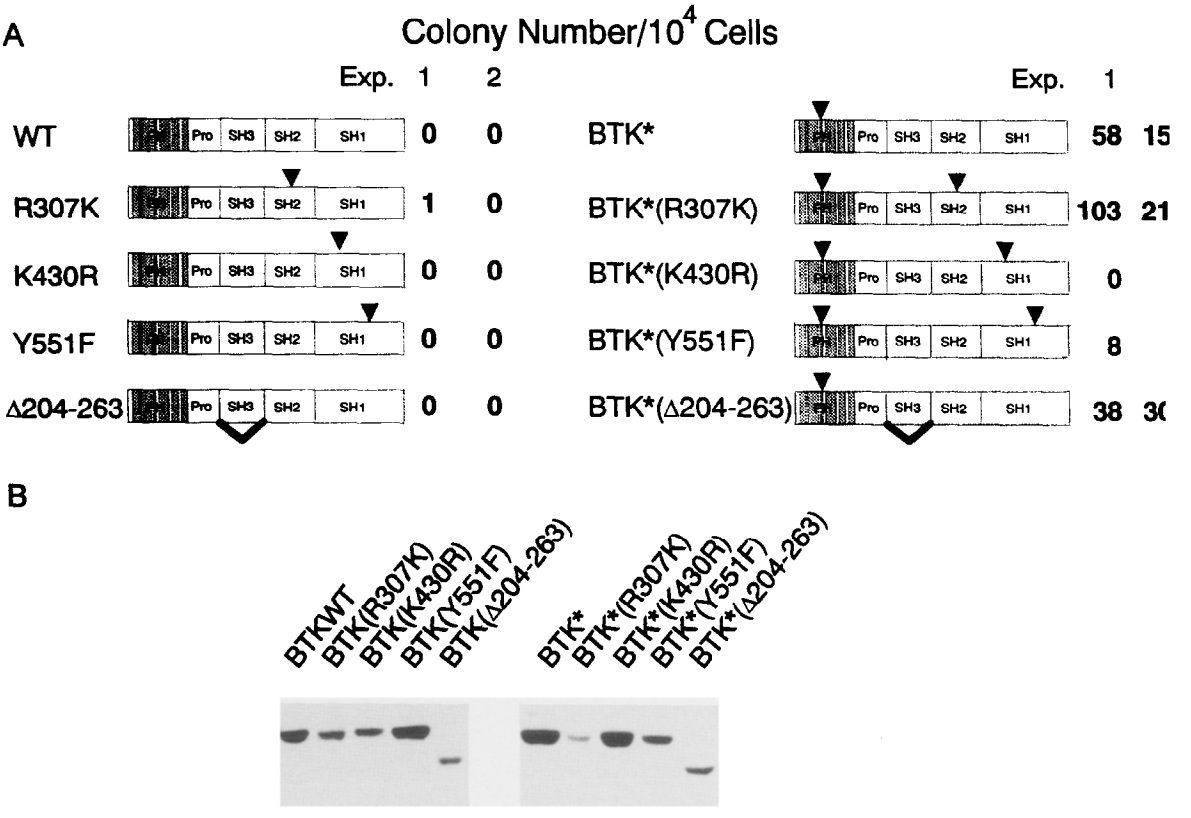


Figure 4. Transformation of NIH 3T3 Cells by BTK* Chimeras

(A) A schematic representation of the BTK mutants and their transformation activities. NIH 3T3 cells (10^4) expressing wild-type or mutants c were plated in soft agar in each 6 cm plate in duplicate. Colonies larger than 0.2 mm diameter were counted 15 days after plating. The nu presented are averages of duplicate plates.

(B) Expression of BTK mutants. NIH 3T3 cells were infected with different BTK viruses as indicated. Infected cells were selected in 500 G418 for 2 weeks. Cells (10^6) were lysed and analyzed by immunoblotting with anti-BTK antiserum (1:500), followed by HRP-conjugate anti-rabbit secondary antibody (1:5,000), and visualized by ECL technique.

protection analysis. BTK retroviral mRNA was expressed in all clones that became IL-5 independent (Figure 7B, top). The amount of RNA in each lane was monitored by the expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene (Figure 7B, bottom).

Discussion

Activation of BTK Provides Insight into Its Regulation

Cytoplasmic tyrosine kinases can be activated by alternative mechanisms. For the Src subfamily, certain mutations within the SH3, SH2, or SH1 regions can be associated with transforming activity (Hirai and Varmus, 1990; O'Brien et al., 1990; Levy and Brugge, 1989; Kato et al., 1986). In addition, deletion or mutation of a negative regulatory phosphorylation site (Tyr527) can lead to up-activation of kinase activity and transforming activity (Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987; Cartwright et al., 1987; Reynolds et al., 1987). In contrast, mutations in the SH3, or SH2 domains of BTK render the protein inactive and are associated with a genetic immunodeficiency (Zhu et al., 1994; Saffran et al., 1994).

The isolation of *BTK** and the identification of the activating mutation occurring within the PH domain (E41K) vides a unique insight for understanding the regulati this new family of the cytoplasmic tyrosine kinases. BTK subfamily of tyrosine kinases has a high degr homology within the PH domain. All members, e DSrc28C, have a glutamic acid at amino acid positic (Musacchio et al., 1993). It remains to be determ whether a similar mutation to BTK* would alter biolo properties of any of the other family members. Struc analysis of PH domains by nuclear magnetic reson or X-ray crystallography suggests that a pocket or cl the amino-terminal region would consist of charged dues including Arg28 and Glu41 as potential ligand ing components (Macias et al., 1994; Ferguson e 1994; Gibson et al., 1994). Alignment of the sequen BTK to the three-dimensional structure of PH dor suggests that the residues involved in the xid mu (Arg28) and BTK* (Glu41) are likely to be in close cc in two antiparallel β sheets. There may be an electro interaction between these two residues in the norm tein important for ligand binding.

An increase in membrane targeting is another po consequence arising from the activation of BTK*. kinases, such as Raf, can be recruited to the mem

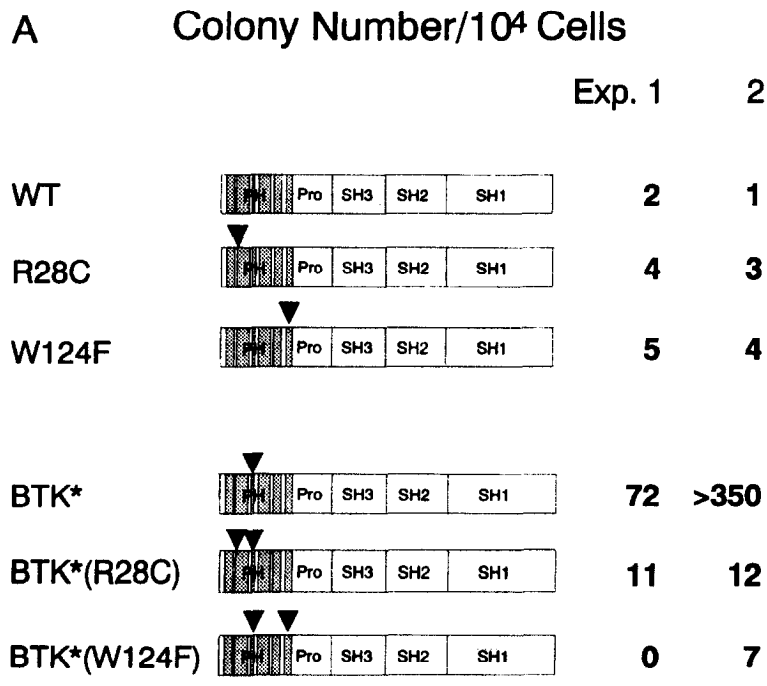
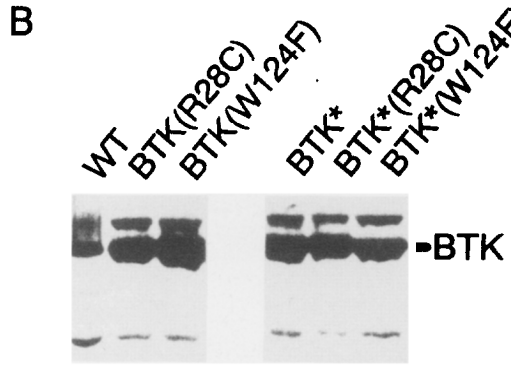


Figure 5. PH Domain Mutants Blocks BTK* Transformation

(A) A schematic representation of the BTK mutants and their transformation activities. NIH 3T3 cells (10^4) expressing wild-type or mutants of BTK were plated in soft agar in each 6 cm plate in duplicate. Colonies larger than 0.2 mm diameter were counted 15 days after plating. The numbers presented are averages of duplicate plates.

(B) Expression of BTK mutants. NIH 3T3 cells were infected with different BTK viruses as indicated. Infected cells were selected in 500 μg/ml G418 for 2 weeks. Cells (10^6) were lysed and analyzed by immunoblotting with anti-BTK antiserum (1:500), followed by HRP-conjugated goat anti-rabbit secondary antibody (1:5,000), and visualized by ECL technique.



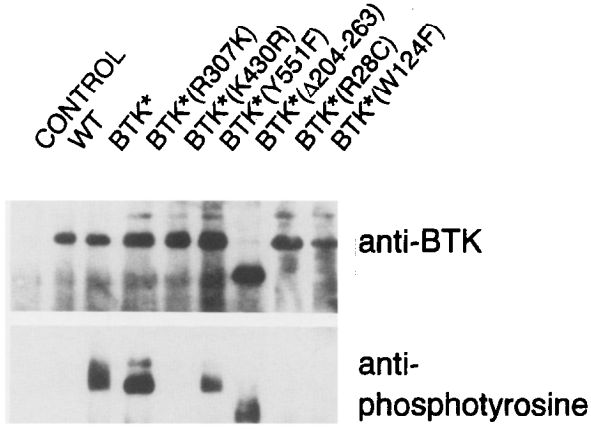


Figure 6. Tyrosine Phosphorylation of BTK* Chimeras

NIH 3T3 cells expressing TK-*neo* (control), wild-type BTK, and BTK mutants were lysed and BTK protein was immunoprecipitated with anti-BTK antiserum. The immunoprecipitates were subjected to 10% SDS–PAGE and analyzed by immunoblotting. The figure shows the immunoprecipitated BTK protein by anti-BTK immunoblotting with anti-BTK antiserum (1:500), followed by HRP-conjugated goat anti-rabbit secondary antibody (1:5,000), and visualized by ECL technique (top), and the in vivo tyrosine phosphorylation of BTK by anti-phosphotyrosine immunoblotting (bottom) with 4G10 antibody (1:1,000), followed by HRP-conjugated goat anti-mouse secondary antibody (1:5,000), and visualized by ECL technique.

by binding to activated partners, like Ras (Traverse et al., 1993). Covalent lipid modifications can bypass this copartner requirement (Stokoe et al., 1994; Leevers et al., 1994). Our data indicates an increased fraction of BTK* is found in the membrane as compared with wild-type BTK. We do not know the precise mechanism for membrane interaction. However, prior work from our lab and others has indicated that Gβγ can interact with the PH domain of BTK in such membrane recruitment (Tsukada et al., 1994; Touhara et al., 1994). Data from other groups suggests that the PH domain can have direct interaction with selected phospholipids, including PIP_2 and D-myoinositol

A

Virus	Positive wells/Total
Neo	4/72
BTK WT	0/72
BTK* STOCK 1	47/72
BTK* STOCK 2	26/72
v-Abl	72/72

B

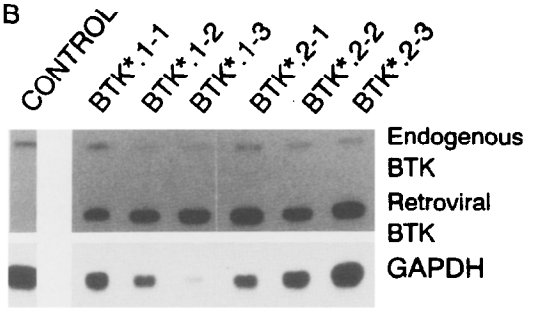


Figure 7. BTK* Renders Y16 Cells Factor Independent

(A) Y16 cells infected with TK–*neo*, wild-type BTK, BTK*, or v-abl viruses of equivalent titer were selected with 500 μg/ml G418 in the presence of 5 U/ml IL-5 for 10 days. Cells (2×10^4) were plated in each of 72 wells on a 96-well plate without IL-5. Wells that became confluent 10 days after plating were scored as positive.

(B) Expression of BTK* in Y16 cells. Of the total RNA from each cell line, 20 μg was subjected to S1 nuclease protection analysis (see Experimental Procedures): Y16 cells infected with TK–*neo* virus and selected with G418 (control) or Y16 cells expanded from factor independent clones from 96-well plates (see 1–1, 1–2, 1–3 for cells initially infected with BTK* stock 1 and 2–1, 2–2, 2–3 for cells initially infected with BTK* stock 2).

1,4,5-trisphosphate (Harian et al., 1994; Cifuentes et al., 1994). The precise relationship between these two sets of observations is not yet clear.

The occurrence of PH domains in signal transduction proteins is clear, but their modes of action are not defined. The inactivating mutations found in xid mice and XLA patients in concert with the activating mutation of BTK* should prove useful in functional analysis of other PH domain–containing proteins.

Involvement of BTK in Multiple B Cell Signaling Pathways

Genetic and biochemical analyses suggest that BTK plays a role in a broad range of B cell signaling pathways. Defective responses to specific growth factors and membrane cross-linking events, such as IL-5 and surface IgM, are well documented through studies of xid mice (Yamashita et al., 1995; Koike et al., 1995; Hasbold and Klaus, 1994; Howard et al., 1993; Hitoshi et al., 1993; Go et al., 1990). The closely related tyrosine kinase ITK has recently been described as playing a role in T cell receptor/CD28 signaling (August et al., 1994). The possible role of BTK in B coreceptor pathways should be vigorously investiga BTK* should prove useful as a constitutively active f of the enzyme that could bypass receptor activation cooperate with downstream signal components.

Our data showing that BTK* can partially relieve grc factor IL-5 dependence for the Y16 cell line is compa with the genetic defect in IL-5 signaling seen in xid r (Hitoshi et al., 1993). However, the relatively weak e of BTK* as compared with a strong oncogene, sucl v-*abl*, suggests that alternative signals must complen that emanating from BTK* or that the quantitative strei of the signal from BTK* may not be sufficient for full gr factor independence. BTK* was unable to transform mature B lymphoid cells directly in in vitro long term b marrow culture systems. (A. S., unpublished d McLaughlin et al., 1987). Further studies to define pathway of action of BTK* will require more direct kn edge of its substrates and genetic cofactors.

Experimental Procedures

NIH 3T3 Cell Culture and Retrovirus-Mediated Gene Transf

NIH 3T3 cells were grown in DMEM media supplemented wit fetal calf serum. Recombinant *BTK* retroviral cDNAs were constr by inserting the wild-type and mutant *BTK* cDNAs int pSRαMSVTK–*neo* vector (Muller et al., 1991). Helper-free retr stocks were prepared by transient hyperexpression (Muller 1991), in which 293T cells were used to increase the viral titer et al., 1993). The titer of the viruses were measured by their ab confer neomycin resistance to NIH 3T3 cells ($\sim 10^6$ cfu/ml). titered viruses were used to infect fibroblast and lymphoid cel rescue the integrated *BTK* gene in NIH 3T3 cells, a replication c tent Moloney MuLV was used to superinfect those cells. The that contains the virus carrying the rescued *BTK* gene was col and used to infect fresh NIH 3T3 cells. Colony-forming abilit measured by an agar assay as described (Lugo and Witte, 1989) NIH 3T3 cells. In brief, 10^4 cells infected with different BTK v were plated in Iscove's media containing 20% fetal calf seru 0.3% noble agar on an agar bed with 0.6% noble agar and m each 6 cm plate. Colonies were scored positive (more than 0 in diameter) 2 weeks after plating.

Y16 Cells and Factor-Independent Assay

Y16 (Takaki et al., 1990) cells were grown in RPMI 1640 media s mented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, U/ml IL-5. Y16 cells (5×10^6) were infected for 3 hr with TI wild-type BTK, and BTK* viruses. Infected cells were selected μg/ml G418 containing media for 10 days. Cells (2×10^4) were in each of 72 wells of a 96-well plate without IL-5. Confluent well counted as factor independent 10 days after plating.

Genomic Screening and Sequencing Analysis of *BTK* cDN

BTK agar colonies were picked and expanded in liquid cultur genomic DNA from cells derived from one such colony was p and cloned into the EcoRI site of the λZAPII/EcoRI/CIAP clor (Stratagene). Library screening followed plaque lifting tech (Sambrook et al., 1989), with a probe of *BTK* cDNA labeled by r priming kit Prime-It II (Stratagene). Positive phage DNAs we verted to phagemid by in vivo excision according to the vendor p (Stratagene). Double-stranded DNA sequencing analysis (Sa al., 1977) was performed on phagemid DNA using primer set lings et al., 1993) that cover the whole *BTK* coding sequence

Mutagenesis

Point mutations BTK(R307K), BTK(K430R), BTK(Y551F), BTK(BTK(W124F) and BTK*(R307K), BTK*(K430R), BTK*(Y BTK*(R28C), BTK*(W124F) were created using site-directe

genesis kit Sculptor (Amersham) and Alter Sites (Promega) respectively. *BTK*(Δ204–263) was constructed by ligating two *BTK* fragments (nucleotides 1–748 and nucleotides 927–1902) into a pBluescriptSK(–) vector (Stratagene), which already has partial *BTK* sequences (nucleotides 1903–2399). These mutants were subcloned into the pSRαMSVTK–*neo* vector (Muller et al., 1991). A HindIII fragment in *BTK** was replaced by that of *BTK*(Δ204–263) to create *BTK**(Δ204–263) in the pSRαMSVTK–*neo* vector.

Protein Analysis

Total cell lysates were prepared by lysing 10^6 cells in 200 μl boiling 2× sample buffer (2% SDS, 0.1 M Tris [pH 6.8], 20% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). Immunoprecipitation and immunoblotting were done as described (Konopka and Witte, 1985). In brief, 10^7 cells were lysed on the plate with boiling cell lysis buffer (1% Triton X-100, 10 mM phosphate buffer [pH 7.0], 150 mM NaCl, 500 μM sodium vanadate) plus 1% SDS. The cell lysates were diluted 10-fold with cell lysis buffer and clarified by ultracentrifugation at 100,000 × g for 30 min at 4°C. The supernatants were incubated with 10 μl anti-BTK serum at 4°C for 2 hr. The samples were mixed with 100 μl protein A–sepharose beads (suspended in 5 beads volume of cell lysis buffer) in the cold room for 1 hr on a nutator and were washed three times with cell lysis buffer. For immunoblotting, immunoprecipitates or cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose filter. The filter was blotted with 5% skim milk in 150 mM NaCl, 10 mM Tris (pH 7.5) and 500 μM sodium vanadate for 1 hr, except for anti-phosphotyrosine blotting, which was blocked with 2% gelatin in 150 mM NaCl, 10 mM Tris (pH 7.5), and 500 μM sodium vanadate at 37°C for 1 hr. The filter was then sequentially blotted with primary antibodies (anti-BTK [Tsukada et al, 1993], 1:500 dilution; anti-GAP [Transduction Laboratories], 1:250 dilution; anti-phosphotyrosine [4G10], 1:1,000 dilution) and secondary antibodies (horseradish peroxidase [HRP]–conjugated goat anti-rabbit or goat anti-mouse antibodies [BioRad], 1:5,000 dilution) according to Konopka and Witte (1985). Proteins were visualized by enhanced chemiluminescence (ECL) technique (Amersham). In vitro autokinase assay was performed as previously described (Konopka and Witte, 1985). In brief, BTK immunoprecipitates were prepared as mentioned above. The kinase reaction was carried out at 25°C for 5 min in a 45 μl final volume with 2 μl [γ-^{32}P] ATP (3,000 Ci/mmol, 5 mCi/ml) and 10-fold excess (at a final concentration of 4×10^{-7} μmol/ul) cold ATP.

Subcellular Fractionation

Subcellular fractionation was performed as described by Kawakami et al. (1994). In brief, 10^7 NIH 3T3 cells expressing wild-type BTK or BTK* were scraped into 1 ml hypotonic lysis buffer (1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 40 ng/ml PMSF) and were incubated on ice for 30 min. Cells were then Dounce homogenized (30 strokes). Cell lysates were loaded onto a 1 M sucrose cushion and spun at 1,600 × g for 10 min. Postnuclei supernatants were spun at 100,000 × g for 30 min. The supernatant contained the cytosolic fraction. The precipitate containing the membrane fraction was washed with the hypotonic lysis buffer twice and resuspended in hypotonic lysis buffer containing 1% SDS in the same volume as the cytosolic fraction. It was designated the membrane fraction.

S1 Nuclease Protection Analysis

S1 nuclease protection assays were performed according to Weaver and Weissmann (1979) using 20 μg of total RNA from the indicated Y16 cell lines. The *BTK* probe corresponded to a 773 bp BglII–KpnI fragment from pBluescriptSK(-) *BTK*, which was 5′ end labeled at the BglII site with [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont/New England Nuclear) and T4 polynucleotide kinase (20 U, Pharmacia). The probe will protect a 723 bp fragment of the endogenous BTK mRNA and a 614 bp fragment of the retrovirally encoded RNA. For the GAPDH probe (Fort et al., 1985), a fragment of rat GAPDH was 5′ end labeled at the NcoI site. RNA samples were incubated overnight at 52°C with 20,000 cpm of probes. The S1 digestion was performed as described (Weaver and Weissmann, 1979). The products of the reaction were electrophoresed on a 5% polyacrylamide sequencing gel and exposed to film for 6 hr.

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